(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/28569 A1

(51) International Patent Classification[7]: A61K 31/70, C07H 21/02, 21/04, C07K 14/00, 16/00, 17/00, C12N 15/09, 15/63, 15/86, 15/87, 15/88

(21) International Application Number: PCT/US00/28516

(22) International Filing Date: 13 October 2000 (13.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/159,782 15 October 1999 (15.10.1999) US

(71) Applicant: CANJI, INC. [US/US]; Richard B. Murphy, 3525 John Hopkins Court, San Diego, CA 92121 (US).

(72) Inventor: MURPHY, Richard; 3974 Caminito Amparo, San Diego, CA 92122 (US).

(74) Agent: SMITH, Timothy; Townsend and Townsend and Crew, LLP, Two Embarcadero Ctr, Suite 800, San Francisco, CA 94111 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/28569 A1

(54) Title: TARGETED VECTORS

(57) Abstract: This invention provides therapeutic and diagnostic agent delivery vehicles, including viral vectors, that are complexed to a targeting moiety by coordinate covalent linkages mediated by a transition metal ion. The complex is typically formed with a transition metal ion that is in a kinetically labile oxidation state; after the complex is formed, the oxidation state of the transition metal ion is changed to one that renders the complex kinetically stable. The use of a coordinate covalent linkage to attach the targeting moiety to the delivery vehicle provides advantages such as the ability to readily attach a different targeting moiety to a delivery vehicle without modifying the delivery vehicle itself. This flexibility is achieved without sacrificing stability of the complex.

71(11):8221-8229 (incorporation of RGD peptides into adenoviral fiber proteins); Arnberg *et al.* (1997) *Virology* 227:239-244 (modification of adenoviral fiber genes to achieve tropism to the eye and genital tract); Harris and Lemoine (1996) *TIG* 12(10):400-405; Stevenson *et al.* (1997) *J. Virol.* 71(6):4782-4790; Michael *et al.* (1995) *Gene Therapy* 2:660-668 (incorporation of gastrin releasing peptide fragment into adenovirus fiber protein); and Ohno *et al.* (1997) *Nature Biotechnology* 15:763-767 (incorporation of Protein A-IgG binding domain into Sindbis virus).

However, the design of a functional chimeric protein for targeting is not facile. For example, if one wishes to create a chimeric adenoviral knob protein containing an targeting domain, the recombinant knob protein must be able to (a) assemble properly into the icosahedral viral structure and (b) also retain the binding specificity of the targeting moiety. This may involve significant and complex molecular modeling to incorporate the targeting moiety into the appropriate region of the knob protein to insure that the targeting moiety is on the surface of the knob protein. Additionally, since the precise process for assembly of the adenoviral particle is poorly understood it is possible that insertion of a large targeting moiety will sufficiently interrupt the three dimensional structure of the viral protein so that it does not efficiently assemble into an infectious virion. Furthermore, whenever one wishes to change the targeting properties of the adenovirus, it is necessary to reengineer the knob protein taking into account all of the foregoing, which can be a lengthy process. Moreover, the manipulation of the adenoviral genome to obtain a gene that encodes the chimeric protein is a time consuming process, due to the size and complexity of the adenoviral genome.

In order to avoid these hurdles, other methods of cell specific targeting rely on the conjugation of antibodies or antibody fragments to the envelope proteins (*see, e.g.* Michael *et al.* (1993) *J. Biol. Chem.* 268:6866-6869, Watkins *et al.* (1997) *Gene Therapy* 4:1004-1012; Douglas *et al.* (1996) *Nature Biotechnology* 14: 1574-1578. This approach also has its limitations. First, in the case of chemically conjugating the antibody (or antibody fragment) to the surface of the virion, the linkage is generally achieved by modification of amino acyl side chains in the antibody (particularly through lysine residues). As it is difficult to control the stoichiometry of this reaction, one can envision the resulting virion being coated with antibodies in a variety of orientations. As the binding specificity of the antibody

SUMMARY OF THE INVENTION

The present invention provides targeted complexes that are useful for delivering molecules to a particular cell or tissue type of interest. The invention provides targeted complexes of the formula:

{(delivery vehicle–CM) – TMI – (CM–targeting ligand)};

The delivery vehicle can be, for example, a peptide vector, a peptide-DNA aggregate, a liposome, a gas-filled microsome, an encapsulated macromolecule, and the like. In some embodiments, the delivery vehicle is a viral vector. Particularly suitable viral vectors include a retrovirus, a vaccinia virus, a herpes virus, an adeno-associated virus, a minute virus of mice (MVM), a human immunodeficiency virus, a sindbis virus, an MoMLV, and a hepatitis virus.

"CM" is a chelating moiety, such as a chelating peptide or an organic chelating agent. TMI is a transition metal ion. CM-targeting ligand is a chelating moiety (CM) covalently linked to a targeting ligand that can bind to a cell or tissue of interest.

The invention also provides methods for producing a kinetically inert targeted delivery vehicle complex. These methods involve: a) preparing a kinetically labile transition metal complex by contacting a delivery vehicle–CM and a CM–targeting ligand with a transition metal ion that is in a kinetically labile oxidation state; and b) changing the oxidation state of the metal ion to form the kinetically inert complex

Also provided by the invention are methods of delivering a therapeutic or diagnostic agent to a target cell in an organism. These methods involve administering to an organism a targeted complex of the formula:

{(delivery vehicle-CM) – TMI – (CM–targeting ligand)};

wherein delivery vehicle-CM is a delivery vehicle that displays on its surface a polypeptide that comprises a chelating moiety (CM), TMI is a transition metal ion, and CM-targeting ligand is a chelating moiety (CM) covalently linked to a targeting ligand that binds to the target cell.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an illustration of one embodiments of the complexes of the present invention. The drawing provides is a diagrammatic representation of a complex wherein the virus is an adenovirus is containing a modified knob domain containing a chelating peptide

the thermodynamic tendency of a species to exist under equilibrium conditions. A kinctically inert complex, on the other hand, is one that is not labile, *i.e.*, a particular complexcd ion is not ablc to readily cngage in rcactions that result in rcplaccmcnt of one or morc ligands in its coordination sphcre by others. For cxample, in an aqueous environmcnt, unoccupied coordination positions on a transition metal ion are occupied by water. A chelating peptide or other chelating agent must displace the water molecules to form a complex. When such reactions occur rapidly, the reaction is termed "labile." However, where such reactions occur very slowly or not at all, the complex is said to be kinetically "inert." Kinetic lability or inertness, unlike thermodynamic stability or instability, is thus related to the reaction rate. A complex can be thermodynamically stable even though the on/off reactions occur very rapidly (*see, e.g., Advanced Inorganic Chemistry*, Cotton, F.A. and Wilkinson, G. (1972) 3rd ed. Interscience Publishers, p.652). Conversely, a complex can be kinetically inert, and thus last for periods of time ranging from days to years, even though the complex is thermodynamically unstable (equilibrium lies in favor of dissociation) because the rate of dissociation is low.

While the affinity of an antibody for a particular protein may be high and the resulting equilibrium constant of this reaction suggests the formation of a "stable" complex, this does not indicate that the complex will be kinetically stable in solution over a period of time. This presents a particularly serious drawback when such non-covalent interactions are used to attach a targeting ligand to a delivery system which is then introduced into a biological system. The increased volume upon introduction of the complex to an organism will result in an equilibrium constant (K_{eq}) favoring dissociation, since the blood volume is essentially infinitely large in comparison to the administered volume. Furthermore, the toxicity of the free components of the complex may provide an additional degree of uncertainty in the use of such complexes in mammalian systems. Since non-covalently linked complexes will necessarily result in free species upon administration to an organism, the toxicity of the free species in addition to the complex would need to be evaluated. In human beings, this would likely complicate the regulatory approval process for such complexes as it would require additional toxicology clinical studies. These problems are avoided by the present invention, which uses a kinetically inert coordinate covalent linkage to attach the targeting ligand to the viral coat protein or other gene delivery system.

virus, adeno-associated virus (*see, e.g.*, Xiao *et al.*, *Brain Res.* 756:76-83 (1997), minute virus of mice (MVM), HIV, sindbis virus, and retroviruses (including but not limited to Rous sarcoma virus), and MoMLV, hepatitis B virus (*see, e.g.*, Ji *et al.*, *J. Viral Hepat.* 4:167-173 (1997). Typically, genes of interest are inserted into such vectors to allow packaging of the gene construct, typically with accompanying viral DNA, followed by infection of a sensitive host cell and expression of the gene of interest. One example of a preferred recombinant viral vector is the adenoviral vector delivery system which has a deletion of the protein IX gene (*see*, International Patent Application WO 95/11984, which is herein incorporated by reference in its entirety for all purposes).

In some instances it may be advantageous to use vectors derived from a different species from that which is to be treated in order to avoid the preexisting immune response. For example, equine herpes virus vectors for human gene therapy are described in WO98/27216 published August 5, 1998. The vectors are described as useful for the treatment of humans as the equine virus is not pathogenic to humans. Similarly, ovine adenoviral vectors may be used in human gene therapy as they are claimed to avoid the antibodies against the human adenoviral vectors. Such vectors are described in WO 97/06826 published April 10, 1997.

The virus can be replication competent (*e.g.*, completely wild-type or essentially wild-type such as Ad dl309 or Ad dl520), conditionally replicating (designed to replicate under certain conditions) or replication deficient (substantially incapable of replication in the absence of a cell line capable of complementing the deleted functions). Alternatively, the viral genome can possess certain modifications to the viral genome to enhance certain desirable properties such as tissue selectivity. For example, deletions in the E1a region of adenovirus result in preferential replication and improved replication in tumor cells. The viral genome can also modified to include therapeutic transgenes (as more fully described below). The virus can possess certain modifications to make it "selectively replicating," *i.e.* that it replicates preferentially in certain cell types or phenotypic cell states, *e.g.*, cancerous. For example, a tumor or tissue specific promoter element can be used to drive expression of early viral genes resulting in a virus which preferentially replicates only in certain cell types. Alternatively, one can employ a pathway-selective promoter active in a normal cell to drive expression of a repressor of viral replication. For example, a

inserts are desired to achieve the therapeutic effect in the target cell, a "gutted" or minimal viral vector system can be employed. Such vectors are well known in the art and a review of this technology is provided in Morsy and Caskey, *Molecular Medicine Today*, Jan. 1999 pp. 18-24; *Zhang, et al.* (WO98/54345A1 published Dec. 3, 1998); and Kochanek *et al.* (1996) *Proc. Nat'l. Acad. Sci. USA* 93: 5731-5736.

In a presently preferred embodiment of the invention, the virus is an adenovirus. The use of adenoviral vectors for the delivery of exogenous transgenes are well known in the art. *See, e.g.*, Zhang, W-W. (1999) *Cancer Gene Therapy* 6:113-138. The term "adenovirus" refers collectively to animal adenoviruses of the genus mastadenovirus including, but not limited to, human, bovine, ovine, equine, canine, porcine, murine and simian adenovirus subgenera. In particular, human adenoviruses include the A-F subgenera as well as the individual serotypes thereof the individual serotypes and A-F subgenera including but not limited to human adenovirus types 1, 2, 3, 4, 4a, 5, 6, 7, 8, 9, 10, 11 (Ad11A and Ad 11P), 12, 13,14,15,16,17,18,19, 19a, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 34a, 35, 35p, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, and 91. The bovine adenoviruses useful in the invention include, but are not limited to, bovine adenovirus types 1,2,3,4,7, and 10. Canine adenoviruses, as used herein, includes but is not limited to canine types 1 (strains CLL, Glaxo, RI261, Utrect, Toronto 26-61) and 2. Equine adenoviruses of interest include, but are not limited to, equine types 1 and 2 and porcine adenoviruses of interest include, for example, porcine types 3 and 4. In a presently preferred practice of the invention, the virus is an adenovirus of serotype 2 or 5.

Adenoviral polypeptides into which one can incorporate a chelating peptide include, for example, the fiber protein (*see, e.g.*, US Patent Nos. 5,846,789, 5,770,442, 5,543,328 and 5,756,086), the penton base protein (*see, e.g.*, US Patent Nos. 5,559,099, 5,731,190 and 5,712,136), and the hexon protein (*see, e.g.*, US Patent No. 5,922,315).

Retroviral vectors can also be targeted using the coordinate covalent complexes of the present invention. The envelope protein of retroviral vectors is modified to include a chelating peptide. The retroviral gene that encodes the *env* polypeptide is modified so that a fusion between a chelating peptide and all or part of the *env* polypeptide is expressed. Modifications of retroviral *env*-encoding genes are described in, for example, US Patent Nos. 5,869,331. US Patent No. 5,736,387 describes the use of chimeric targeting

also has reduced or eliminated affinity for the natural target cell. Similarly, infection of adenoviruses into susceptible cells involves the binding of the adenovirus fiber protein (in particular, the C-terminal knob domain) to the coxsackievirus and adenovirus receptor (CAR), which serves as the primary cellular receptor. The subsequent internalization of the virion involves Arg-Gly-Asp (RGD) sequences in the penton base, which interact with the secondary host cell receptors, integrins $\alpha_v\beta 3$ and $\alpha_v\beta_5$. Thus, by disrupting either or both of the fiber protein and the penton base, one can eliminate the native tropicity of the adenoviral vector (*see, e.g.,* Douglas *et al.* (1999) *Nature Biotechnology* 17: 470-475; US Patent No. 5,885,808). The disruption of proteins involved in native viral tropism can be as an intended consequence of the introduction of the chelating peptide, or can be accomplished by other manipulations of the viral genome. Parvoviral vectors are another example of viral vectors that can be targeted using the modified coat protein-chelating peptide complexed to a targeting ligand.

The invention also provides complexes in which a conformationally restrained non-native amino acid sequence is attached to a surface-displayed chelating moiety. Conformationally constrained peptides are generally more effective in targeting delivery to specific cells and/or tissues than unconstrained peptides. US Patent No. 6,057,155 describes the use of such conformationally-restrained, or "constrained" amino acid sequences in a chimeric adenovirus fiber protein. The ability of the chimeric fiber protein to bind to the cell and/or mediate cell entry is increased, *e.g.*, relative to the wild-type protein. According to US Patent No. 6,057,155, the conformational constraint can be achieved by placing a nonnative amino acid sequence in an exposed loop of the chimeric fiber protein, or, through the placement of the sequence in another location and creation of a loop-like structure comprising the nonnative amino acid sequence at that site. The present invention facilitates making the chimeric fiber protein by eliminating the need to alter the viral genome in order to introduce the nonnative amino acid sequence. Rather, a polypeptide that includes a chelating moiety and the nonnative amino acid sequence and associated loop structure is made by, for example, recombinant expression. This polypeptide is then attached to a viral vector that displays a corresponding chelating moiety through a transition metal ion.

liposome structure need be developed; once such structures having chelating moieties are made, it is a simple matter to attach a desired targeting ligand. It is not necessary to reengineer a liposome-anchored polypeptide or other anchoring moiety for each of the targeting moieties that are of interest.

Coordinate covalent linkages are also useful for attaching targeting moieties to other vehicles for delivering nucleic acids or other compounds. For example, one can use these linkages to attach a targeting moiety to a polycation, which is in turn complexed with a nucleic acid that is to be targeted to a particular cell or tissue (*see, e.g.,* US Patent Nos. 5,874,297, 5,166,320, and 5,635,383). For example, gene constructs or other agents can be conjugated to a cell receptor ligand for facilitated uptake (*e.g.*, invagination of coated pits and internalization of the endosome; *see, e.g.,* Wu *et al.* (1988) *J. Biol. Chem.* 263:14621-14624; WO 92/06180; US Patent No. 5,871,727) through a coordinate covalent linkage. Again, the use of coordinate covalent attachment simplifies the attachment of the targeting ligand molecules to the delivery vehicle.

Other suitable delivery systems include, but are not limited to, an HVJ (Sendai virus)-liposome gene delivery system (*see, e.g.*, Kaneda *et al.*, *Ann. N.Y. Acad. Sci.* 811:299-308 (1997)); a "peptide vector" (*see, e.g.*, Vidal *et al.*, *CR Acad. Sci III* 32:279-287 (1997)); a peptide-DNA aggregate (*see, e.g.*, Niidome *et al.*, *J. Biol. Chem.* 272:15307-15312 (1997)); lipidic vector systems (*see, e.g.*, Lee *et al.*, *Crit Rev Ther Drug Carrier Syst.* 14:173-206 (1997)); polymer coated liposomes (Marin *et al.*, United States Patent No. 5,213,804, issued May 25, 1993; Woodle *et al.*, United States Patent No. 5,013,556, issued May 7, 1991); cationic liposomes (Epand *et al.*, United States Patent No. 5,283,185, issued February 1, 1994; Jessee, J.A., United States Patent No. 5,578,475, issued November 26, 1996; Rose *et al*, United States Patent No. 5,279,833, issued January 18, 1994; Gebeyehu *et al.*, United States Patent No. 5,334,761, issued August 2, 1994); gas filled microspheres (Unger *et al.*, United States Patent No. 5,542,935, issued August 6, 1996), encapsulated macromolecules (Low *et al.* United States Patent No. 5,108,921, issued April 28, 1992; Curiel *et al.*, United States Patent No. 5,521,291, issued May 28, 1996; Groman *et al.*, United States Patent No. 5,554,386, issued September 10, 1996; Wu *et al.*, United States Patent No. 5,166,320, issued November 24, 1992). In each case, the transition metal ion-

the amino or carboxy terminus of the protein or can be incorporated internally into the delivery vehicle protein in an surface-exposed domain of the protein.

Examples of an adenovirus in which the knob protein has been modified to contain a metal chelating peptide are known in the art. For example, Douglas *et al.* describe a recombinant adenovirus in which a poly-His metal chelating peptide has been incorporated into the carboxy terminal domain of the adenoviral fiber protein (*Nature Biotechnology* (1999) 17: 470-475). The penton and hexon polypeptides are also suitable adenovirus coat proteins for introduction of the chelating peptide. Apart from the insertion of the metal chelating peptide in the coat protein, the remainder of the viral genome can be wild-type or can be modified through conventional recombinant DNA techniques to possess specific properties.

Chelating peptides that are useful in the targeted vectors of the invention include, for example, a polyhistidine sequence. Generally, at least two histidine residues are required to obtain binding to a transition metal ion; the use of additional adjacent histidines increases the binding affinity. Typically, six adjacent histidines are used, although one can use more or less than six. Suitable polyhistidine peptides are described in, for example, Anderson *et al.* (US Patent No. 5,439,829, issued August 8, 1995), Doebli *et al.* (US Patent No. 5,284,993, issued February 8, 1994) and Doebli *et al.* (US 5,310,663, issued May 10, 1994).

In presently preferred embodiments, a nucleotide sequence that encodes a chelating peptide is incorporated into a gene that encodes a polypeptide that is displayed on the surface of a delivery vehicle, and/or the peptidyl targeting ligand. This typically involves constructing a fusion gene in which a nucleic acid that codes for the polypeptide is linked, in reading frame, to a nucleic acid that codes for the chelating peptide. In regard to coat proteins of a virus, the nucleic acid encoding the chelating peptide is preferably placed at a location in the surface polypeptide gene that does not disrupt the ability of the fusion protein obtained to be displayed on the surface of the delivery vehicle. Where the targeting ligand is an antibody, the chelating peptide-encoding nucleic acid can be placed at or near the region of the antibody gene that encodes the carboxyl terminus of either the light chain or the heavy chain, or both.

Cr(III), V(II), Mn(IV) and the low spin forms of Co(III), Fe(II), Ru(II), Os(II), Rh(III), Ir(III), Pd(IV), and Pt(IV) tend to be extremely inert and useful in the practice of the instant invention. Hanzik, Robert P. in *Inorganic Aspects of Biological and Organic Chemistry*, Academic Press, New York, 1976, p. 109. *See also*, Cotton, F. A. and Wilkinson, G. *supra*. In the preferred practice of the invention the metal ion is selected from the group comprising Te, Co, Cr, and Ru. In the most preferred practice of the invention the metal ion is Co. In the most preferred practice of the invention it is desirable to proceed from Co(II), Cr(II), or Ru(III) to Co(III), Cr(III), or Ru(II) respectively to form the inert complex. Producing the necessary change in the oxidation state of the metal ion can be achieved by a variety of redox reagents. For example, oxidizing agents such as oxygen, hydrogen peroxide, and peracids can be used in the practice of the invention. Examples of reducing agents include, for example, thiols, potassium ferrocyanide, potassium thiocyanate, sulfites, and sodium dithionite. These will be prepared in aqueous solutions of appropriate concentrations.

In some instances, one may wish to incorporate a metal ion which is readily detected by diagnostic testing equipment such as x-ray or magnetic resonance imaging. In this manner, a clinician can non-invasively follow the trafficking of the complex within an organism. Additionally, certain heavy metals such as Te^{99} provide therapeutic (*i.e.*, anti-tumor) effects and can be used to complement the efficacy of the vector.

D. Targeting Ligand

The term "targeting ligand" refers to molecules that interact with and bind to cell type surface ligands of particular cells. Examples of such targeting moieties include antibodies against cell surface proteins and ligands for cell surface proteins. Examples of cell surface proteins include tumor antigens, hormone receptors, G-protein coupled receptors, cytokine receptors, and the like.

1. Antibody

In some embodiments, the targeting ligand includes all or part of an antibody that binds to the desired target tissue or cell. The term "antibody" a term used to collectively describe antibodies, fragments of antibodies (such as, but not limited to, Fab, Fab', Fab_2' and Fv fragments), chimeric, humanized, or CDR-grafted antibodies capable of binding antigens of a similar chain polypeptide binding molecules" as described in PCT Application No.

available or can be prepared through conventional techniques used for the generation of antibodies.

3. Ligands for Cell Surface Receptors/Proteins

Nearly every cell type in a tissue in a mammalian organism possesses some unique cell surface receptor, *e.g.*, G-protein coupled receptors. Consequently, when targeting delivery of the complex to a particular cell type, it is possible to incorporate nearly any ligand for the cell surface receptor as a targeting ligand into the complex. For example, peptidyl hormones can be used a targeting moieties to target delivery to those cells which possess receptors for such hormones. Chemokines and cytokines can similarly be employed as targeting ligands to target delivery of the complex to their target cells. A variety of technologies have been developed to identify genes that are preferentially expressed in certain cells or cell states and one of skill in the art can employ such technology to identify ligands which are preferentially or uniquely expressed on the target tissue of interest. When the ligand is a non-peptidyl or non-protein ligand, it is preferred to employ an organic chelating agent covalently linked to the ligand. When the ligand is a protein or peptide, it is preferred that the chelating agent is a chelating peptide. Again, the chelating peptide can be incorporated at any convenient non-essential domain of the ligand. The preparation of recombinant proteins comprising chelating peptides is well known in the art and commercial vectors are available to facilitate the recombinant production of proteins incorporating chelating peptides such as the pBlueBacHis2 vector commercially available from Invitrogen, San Diego, CA.

4. Other Ligands

Other suitable ligands include "totally synthetic affinity reagents," which are described in US Patent Nos. 5,948,635, 5,852,167 and 5,844,076. Binding polypeptides obtained by directed evolution, for example, as described in US Patent No. 5,837,500 can also be used.

Nuclear localization sequences (NLS) can also be attached to a vector using transition metal ion chelating methods of the invention. NLS facilitate trafficking of proteins into a cell nucleus. *See, e.g.,* WO 96/41606 and US Patent No. 6,054,312.

or both species can be exposed to the metal ion in a single reaction vessel. However, in order to maximize the yield and avoid the formation of homogenous polymers of delivery vehicle or dimers of targeting ligand-CM species, it is preferred that the metal ion be exposed to the targeting ligand, excess metal removed, and the targeting ligand containing the kinetically labile metal be exposed to the delivery vehicle containing the modified viral coat protein. Adding the metal to, for example, a viral vector first will likely result in polymerization of the viral particles and precipitation.

The formation of the kinetically inert complex can be achieved using a variety of oxidizing or reducing agents as described above and will depend on the nature of the metal ion involved. Care should be taken to use any particularly harsh conditions which would result in denaturing of the targeting ligand or CM-delivery vehicles.

The purification of the complexes can be accomplished using conventional chromatographic techniques. Preferably, the purification/isolation of the kinetically inert complexes should be performed in the presence of imidazole or a similar agent capable of competing with the formation of a kinetically labile intermediate. This will facilitate the purification of only kinetically inert complexes by disrupting kinetically labile complexes, thus insuring a homogenous kinetically inert complex.

III. Uses of the Targeted Complexes

The complexes of the present invention find use in a wide variety of applications. Among these applications are the targeting of therapeutic or diagnostic agents to particular cells or tissues.

A. Therapeutic Applications

The complexes of the present invention are useful in the treatment of a wide range of diseases in mammalian organisms. The term "mammalian organism" includes, but is not limited to, humans, pigs, horses, cattle, dogs, cats, and the like. In these embodiments, a therapeutic agent is carried in, or attached to, a viral vector, liposome, or other delivery vehicle, to which is complexed the targeting ligand through a transition metal ion.

The methods and compositions of the present invention can be used for the treatment of a variety of maladies common in mammalian organisms. For example, the formulations and methods of the present invention can be used for the treatment of a variety

sequence. Operably linked means that the nucleotide sequences being linked are typically contiguous. However, as enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not directly flanked and may even function in trans from a different allele or chromosome.

Expression of a nucleic acid, such as the production of a polypeptide or an antisense nucleic acid, is desired for many applications. Expression is typically accomplished by placing the nucleic acid to be expressed in an "expression cassette," which is a nucleic acid construct, generated recombinantly or synthetically, that includes nucleic acid elements that are capable of effecting expression of a structural gene in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a desired polypeptide), and a promoter. Additional factors necessary or helpful in effecting expression may also be used as described herein. For example, an expression cassette can also include nucleotide sequences that encode a signal sequence that directs secretion of an expressed protein from the host cell. Transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

In order to effect expression of a nucleic acid of interest, the nucleic acid is operably linked to a promoter sequence operable in the mammal cell. Examples of promoters include, for example, viral promoters endogenous to genome of a viral vector, or promoters derived from other sources. The term "promoter" is used in its conventional sense to refer to a nucleotide sequence at which the initiation and rate of transcription of a coding sequence is controlled. The promoter contains the site at which RNA polymerase binds and also contains sites for the binding of regulatory factors (such as repressors or transcription factors). Promoters can be naturally occurring or synthetic. The promoters can be endogenous to the virus or derived from other sources. The promoter can be constitutively active, or temporally controlled (temporal promoters), activated in response to external stimuli (inducible), active in particular cell type or cell state (selective) constitutive promoters, temporal viral promoters or regulable promoters.

Dreher *et al.* (1997) *J. Biol. Chem.* 272(46); 29364-29371. Examples of radiation inducible promoters are described in Manome *et al.* (1998) *Human Gene Therapy* 9:1409-1417).

b. Therapeutic transgenes

The term "therapeutic transgene" refers to a nucleotide sequence the expression of which in the target cell produces a therapeutic effect. The term therapeutic transgene includes but is not limited to tumor suppressor genes, antigenic genes, cytotoxic genes, cytostatic genes, pro-drug activating genes, apoptotic genes, pharmaceutical genes or anti-angiogenic genes. The vectors of the present invention may be used to produce one or more therapeutic transgenes, either in tandem through the use of IRES elements or through independently regulated promoters.

1) Tumor Suppressor Genes

The term "tumor suppressor gene" refers to a nucleotide sequence, the expression of which in the target cell is capable of suppressing the neoplastic phenotype and/or inducing apoptosis. Examples of tumor suppressor genes useful in the practice of the present invention include the p53 gene, the APC gene, the DPC-4/Smad4 gene, the BRCA-1 gene, the BRCA-2 gene, the WT-1 gene, the retinoblastoma gene (Lee *et al.* (1987) *Nature* 329:642), the MMAC-1 gene, the adenomatous polyposis coli protein (Albertsen *et al.*, United States Patent 5,783,666 issued July 21, 1998), the deleted in colon carcinoma (DCC) gene, the MMSC-2 gene, the NF-1 gene, nasopharyngeal carcinoma tumor suppressor gene that maps at chromosome 3p21.3 (Cheng *et al.* (1998) *Proc. Nat'l. Acad. Sci. USA* 95:3042-3047), the MTS1 gene, the CDK4 gene, the NF-1 gene, the NF2 gene, and the VHL gene.

2) Antigenic Genes

The term "antigenic genes" refers to a nucleotide sequence, the expression of which in the target cells results in the production of a cell surface antigenic protein capable of recognition by the immune system. Examples of antigenic genes include carcinoembryonic antigen (CEA), p53 (as described in Levine, A. PCT International Publication No. WO94/02167 published February 3, 1994). In order to facilitate immune recognition, the antigenic gene may be fused to the MHC class I antigen.

regulated by an exogenous substance such as through the use of the GeneSwitch™ regulatory system (GeneMedicine, Inc. Woodlands, TX).

6) Chemokine Genes

The term "chemokine gene" refers to a nucleotide sequence, the expression of which in a cell produces a cytokine. The term chemokine refers to a group of structurally related low-molecular cytokines weight factors secreted by cells are structurally related having mitogenic, chemotactic or inflammatory activities. They are primarily cationic proteins of 70 to 100 amino acid residues that share four conserved cysteine residues. These proteins can be sorted into two groups based on the spacing of the two amino-terminal cysteines. In the first group, the two cysteines are separated by a single residue (C-x-C), while in the second group, they are adjacent (C-C). Examples of member of the 'C-x-C' chemokines include but are not limited to platelet factor 4 (PF4), platelet basic protein (PBP), interleukin-8 (IL-8), melanoma growth stimulatory activity protein (MGSA), macrophage inflammatory protein 2 (MIP-2), mouse Mig (m119), chicken 9E3 (or pCEF-4), pig alveolar macrophage chemotactic factors I and I (AMCF-I and -II), pre-B cell growth stimulating factor (PBSF),and IP10. Examples of members of the 'C-C' group include but are not limited to monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 2 (MCP-2), monocyte chemotactic protein 3 (MCP-3), monocyte chemotactic protein 4 (MCP-4), macrophage inflammatory protein 1 α (MIP-1-α), macrophage inflammatory protein 1 β (MIP-1-β), macrophage inflammatory protein 1 γ (MIP-1-γ), macrophage inflammatory protein 3-α (MIP-3-α, macrophage inflammatory protein 3 β (MIP-3-β), chemokine (ELC), macrophage inflammatory protein 4 (MIP-4), macrophage inflammatory protein 5 (MIP-5), LD78 β, RANTES, SIS-epsilon (p500), thymus and activation-regulated chemokine (TARC), eotaxin, I-309, human protein HCC-1/NCC-2, human protein HCC-3, mouse protein C10.

7) Pharmaceutical Protein Genes

The term "pharmaceutical protein gene" refers to nucleotide sequence, the expression of which results in the production of protein have pharmaceutically effect in the target cell. Examples of such pharmaceutical genes include the proinsulin gene and analogs (as described in PCT International Patent Application No. WO98/31397, growth hormone

Also of interest are angiogenesis-inducing genes that encode, for example, vascular endothelial growth factor, and other polypeptides that induce angiogenesis. Such genes are useful for treating ischemia and other vascular disorders.

It will be readily apparent to those of skill in the art that modifications and or deletions to the above referenced genes so as to encode functional subfragments of the wild type protein may be readily adapted for use in the practice of the present invention. For example, the reference to the p53 gene includes not only the wild type protein but also modified p53 proteins. Examples of such modified p53 proteins include modifications to p53 to increase nuclear retention, deletions such as the delta13-19 amino acids to eliminate the calpain consensus cleavage site, modifications to the oligomerization domains (as described in Bracco *et al.* PCT published application WO97/0492 or United States Patent No. 5,573,925).

Furthermore, the above therapeutic genes can be secreted into the media or localized to particular intracellular locations by inclusion of a targeting ligand such as a signal peptide or nuclear localization signal (NLS). Also included in the definition of therapeutic transgene are fusion proteins of the therapeutic transgene with the herpes simplex virus type 1 (HSV-1) structural protein, VP22. Fusion proteins containing the VP22 signal, when synthesized in an infected cell, are exported out of the infected cell and efficiently enter surrounding non-infected cells to a diameter of approximately 16 cells wide. This system is particularly useful in conjunction with transcriptionally active proteins (*e.g.* p53) as the fusion proteins are efficiently transported to the nuclei of the surrounding cells. *See, e.g.*, Elliott, G. & O'Hare, P. (1997) *Cell* 88:223-233; Marshall, A. & Castellino, A. (1997) *Nature Biotechnology* 15:205; O'Hare *et al.* PCT publication WO97/05265 published February 13, 1997. A similar NLS derived from the HIV Tat protein is also described in Vives *et al.* (1997) *J. Biol. Chem.* 272:16010-16017.

Additionally, it will be readily apparent to those of skill in the art that a viral or other vector can be engineered to encode more than one therapeutic transgene. The transgenes can be the same (for example to increase the effective gene dosage) or different to achieve complementary effects. Each transgene can be under control of the same promoter (for example through the use of IRES elements) or different promoters. In those situations where it is desirable to produce a vector containing multiple transgenes, it is preferred to use

isothiocynate (TRITC), etc.), digoxigenin, biotin, phycoerythrin, AMCA, CyDyes", and the like), radiolabels (*e.g.*, 3H, 125I, 35S, 14C, 32P, 33P, etc.), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase etc.), spectral colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, etc.) beads. The label may be coupled directly or indirectly to a component of the detection assay (*e.g.*, the detection reagent) according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Preferred labels include those that use: 1) chemiluminescence (using horseradish peroxidase or luciferase) with substrates that produce photons as breakdown products as described above) with kits being available, *e.g.*, from Molecular Probes, Amersham, Boehringer-Mannheim, and Life Technologies/Gibco BRL; 2) color production (using both horseradish peroxidase and/or alkaline phosphatase with substrates that produce a colored precipitate [kits available from Life Technologies/Gibco BRL, and Boehringer-Mannheim]); 3) hemifluorescence using, *e.g.*, alkaline phosphatase and the substrate AttoPhos [Amersham] or other substrates that produce fluorescent products, 4) fluorescence (*e.g.*, using Cy-5 [Amersham]), fluorescein, and other fluorescent tags]; 5) radioactivity. Other methods for labeling and detection will be readily apparent to one skilled in the art.

Preferred enzymes that can be conjugated to targeting ligands using the coordinate covalent linkages of the invention include, *e.g.*, luciferase, and horse radish peroxidase. The chemiluminescent substrate for luciferase is luciferin. Embodiments of alkaline phosphatase substrates include p-nitrophenyl phosphate (pNPP), which is detected with a spectrophotometer; 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) and fast red/napthol AS-TR phosphate, which are detected visually; and 4-methoxy-4-(3-phosphonophenyl) spiro[1,2-dioxetane-3,2'-adamantane], which is detected with a luminometer. Embodiments of horse radish peroxidase substrates include 2,2'azino-bis(3-ethylbenzthiazoline-6 sulfonic acid) (ABTS), 5-aminosalicylic acid (5AS), o-dianisidine, and o-phenylenediamine (OPD), which are detected with a spectrophotometer; and 3,3,5,5'-tetramethylbenzidine (TMB), 3,3'diaminobenzidine (DAB), 3-amino-9-

sterile solution prior to administration. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The formulations can also include delivery enhancing agents to increase uptake of the targeted complexes into the target cells. The terms "delivery enhancers" or "delivery enhancing agents" are used interchangeably herein and includes agents that facilitate the transfer of the nucleic acid or protein molecule to the target cell. Examples of such delivery enhancing agents detergents, alcohols, glycols, surfactants, bile salts, heparin antagonists, cyclooxygenase inhibitors, hypertonic salt solutions, and acetates. Suitable alcohols include for example the aliphatic alcohols such as ethanol, N-propanol, isopropanol, butyl alcohol, acetyl alcohol. Glycols include glycerine, propyleneglycol, polyethyleneglycol and other low molecular weight glycols such as glycerol and thioglycerol. Acetates such as acetic acid, gluconic acid, and sodium acetate are further examples of delivery-enhancing agents. Hypertonic salt solutions like 1M NaCl are also examples of delivery-enhancing agents. Bile salts such as taurocholate, sodium tauro-deoxycholate, deoxycholate, chenodesoxycholate, glycocholic acid, glycochenodeoxycholic acid and other astringents such as silver nitrate can be used. Heparin-antagonists like quaternary amines such as protamine sulfate can also be used. Anionic, cationic, zwitterionic, and nonionic detergents can also be employed to enhance gene transfer. Exemplary detergents include but are not limited to taurocholate, deoxycholate, taurodeoxycholate, cetylpyridium, benalkonium chloride, Zwittergent 3-14 detergent, CHAPS (3-[(3-Cholamidopropyl) dimethylammoniol]-1-propanesulfonate hydrate), Big CHAP, Deoxy Big CHAP, Triton-X-100 detergent, C12E8, Octyl-B-D-Glucopyranoside, PLURONIC- F68 detergent, Tween 20 detergent, and TWEEN 80 detergent (CalBiochem Biochemicals). Particularly preferred delivery enhancing reagents are derivatives of particular impurities that are found in some preparations of Big CHAP; these derivatives are described in PCT Application No. US98/14241 (published January 21, 1999 as WO99/02191).

transgene, expression in addition to more common factors such as the patient's age, weight, sex, physical condition, etc. However, the determination of appropriate dose is a matter of routine experimentation to those of skill in the art. Dose escalation trials in mammalian species generally are initially carried out in small animal species such as swine, eventually in primates. Phase I clinical trials in human beings also include such dose escalation and toxicity assessments. Although such experiments are time-consuming, the skill necessary to achieve the clinically relevant dosage range is a matter of routine experimentation.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

11. The complex of claim 9, wherein the adenovirus is a selectively replicating adenovirus.

12. The complex of claim 7, wherein the adenovirus is replication deficient.

13. The complex of claim 12, wherein the genome of the adenovirus comprises a partial or total deletion of the adenoviral E1 region.

14. The complex of claim 12, wherein the genome of the adenovirus comprises a partial or total deletion of the protein IX-encoding region.

15. The complex of claim 2, wherein the virus is selected from the group consisting of a retrovirus, a vaccinia virus, a herpes virus, an adeno-associated virus, a minute virus of mice (MVM), a human immunodeficiency virus, a sindbis virus, an MoMLV, and a hepatitis virus.

16. The complex of claim 1, wherein the delivery vehicle is selected from the group consisting of a bacteriophage, a peptide vector, a peptide-DNA aggregate, a liposome, a gas-filled microsome, and an encapsulated macromolecule.

17. The complex of claim 1, wherein the targeting ligand is an antibody.

18. The complex of claim 17, wherein the antibody is reactive with a tumor antigen.

19. The complex of claim 17, wherein the antibody is selected from the group consisting of Fab, Fab', Fab_2' and Fv fragments.

20. The complex of claim 17, wherein the antibody is a human antibody.

21. The complex of claim 17, wherein the antibody is a single chain antibody.

a) preparing a kinctically labilc transition mctal complcx by contacting a delivery vehicle–CM and a CM–targeting ligand with a transition metal ion that is in a kinetically labile oxidation state; and

b) changing the oxidation state of thc metal ion to form the kinctically inert complex.

32. The method of claim **31,** wherein the kinetically labile transition metal complex is prepared by:

a) contacting the CM–targeting ligand with the transition metal ion in a reaction vessel and allowing the transition metal ion to bind to the CM to form a transition metal ion–CM–targeting ligand complex;

b) removing uncomplexed transition metal ion from the reaction vessel; and

c) contacting the transition metal ion–CM–targeting ligand complex with the delivery vehicle–CM and allowing the transition metal ion to bind to the CM to form the complex.

33. The method of claim **31,** wherein the kinetically labile transition metal complex is prepared by contacting the CM–targeting ligand and the delivery vehicle–CM with the transition metal ion simultaneously.

34. A method of delivering a therapeutic or diagnostic agent to a target cell in an organism, the method comprising administering to an organism a targeted complex of the formula:

{(delivery vehicle-CM) – TMI – (CM–targeting ligand)};

wherein delivery vehicle-CM is a delivery vehicle that displays on its surface a polypeptide that comprises a chelating moiety (CM), TMI is a transition metal ion, and CM-targeting ligand is a chelating moiety (CM) covalently linked to a targeting ligand that binds to the target cell.

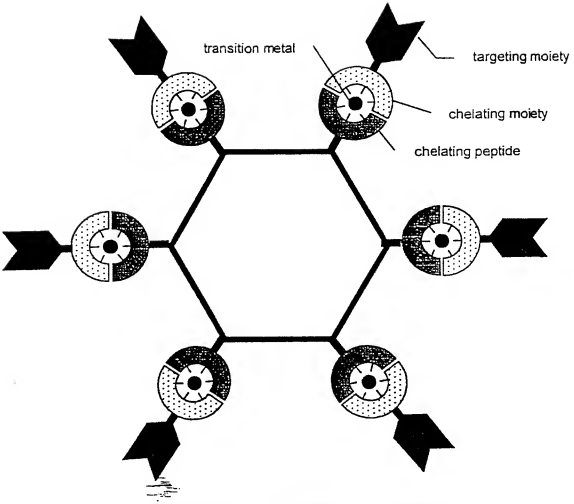
transition metal
targeting moiety
chelating moiety
chelating peptide

Figure 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28516

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.
US CL. : Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/173.3, 173.4, 320.1, 455, 456, 458; 514/44; 530/350, 387.1, 387.7, 388.15; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,439,829 A (ANDERSON et al) 08 August 1995, col. 2, lines 1-8 and 24-33, col. 8, line 10 to col. 15, line 63, col. 30, line 21 to col. 31, line 33, col. 53 line 49 to col. 54, line 8, col. 55, line 1 to col. 56, line 35.	1, 17-22, 25-27, 31-33 ---- 2-16, 23, 24, 28-30
Y	US 5,885,808 A (SPOONER et al) 23 March 1999, col. 2, line 30 to col. 5, line 52, col. 7, line 7 to col. 8, line 19, col. 10, line 44 to col. 11, line 31, col. 13, lines 24-61, Figure 1 and Tables 1 and 2.	2-13, 15, 17-22, 30
Y	US 5,965,541 A (WICKHAM et al) 12 October 1999, col. 5, lines 28-44, col. 9, line 49 to col. 12, line 60, col. 16, line 30-55.	2-5, 7-13, 15, 16, 28-30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 DECEMBER 2000	16 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JANET M. KERR TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28516

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 31/70; C07H 21/02, 21/04; C07K 14/00, 16/00, 17/00; C12N 15/09, 15/63, 15/86, 15/87, 15/88

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/173.3, 173.4, 320.1, 455, 456, 458; 514/44; 530/350, 387.1, 387.7, 388.15; 536/23.1, 23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, DERWENT, MEDLINE, EMBASE, BIOSIS, INPADOC, CAPLUS
search terms: delivery vehicle, delivery complex, chelating peptide, targeting, viral, virus, adenovirus, bacteriophage, antibody, tumor antigen, peptide dna aggregate, liposome, carcinoembryonic antigen, polynucleotide, chelator, chelating, ligand, replication competent, replication deficient, protein IX, transition metal ion, iron, zinc, copper, paramyxovirus, targeting complex, peptide vector, fiber protein.